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A thermodynamic approach to assess a cellular mechanism of inactivation and the thermal resistance of *Listeria innocua*

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Abstract

In order to link kinetic data of isothermally inactivated planktonic cells to the enthalpy changes observed in pellet analysed in the DSC, two strains of *Listeria innocua* were investigated. Isothermal inactivation of planktonic cells showed *z*-values of 4.71°C for *L. innocua* ATCC 33090 and 5.15°C for CCUG 35613(= ATCC 51742). The results of the DSC analyses showed a linearly decreasing remaining enthalpy with increasing dynamic preheating temperature (from 20 to 66-80°C). Deriving equivalent isothermal temperatures, the temperature profiles could be directly correlated with the microbial enthalpy changes, indicating that ribosome denaturation is not the inactivation mechanism for the low thermal treatments studied.

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Keywords: Listeria; inactivation; Differential Scanning Calorimetry; ribosome denaturation

1. Introduction

For the food industry *L. monocytogenes* remains a challenging pathogenic organism and it is a target for control in many ready to eat food products [3]. It has also been recommended as a target organism for thermal processing [1], for which a 6 log reduction (e.g. 70ºC/2 min) is generally advised, also for seafood products [1]. Its thermal resistance has been investigated in different foods [4] and the environmental conditions during heating can have significant effect on the inactivation. Important factors in the food product itself, are water activity, acidity and the presence of other inhibitors [5]. The *z*-value

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has been found to be higher in salmon (fatty species) than in cod (lean species), indicating an influence of fat content of the food matrix on the thermal resistance of *L. monocytogenes* [6].

The exact prime cause of cell death related to heat exposure is not clearly understood [7]. It may vary with the severity of the stress; e.g., mild heat has been observed to cause membrane damage, and leakage of solutes to correlate with loss of viability [8]. At higher temperature, protein denaturation may be a major factor in cell death. Denaturation of the ribosome is recognised as an important inactivation mechanism of Gram-positive bacteria. The link between this mechanism and inactivation of *Listeria* strains has been investigated [9-11]. *L. innocua* is more heat resistant than *L. monocytogenes* and has been found to be well suited surrogate for thermal inactivation processes [12].

The objective of this study is to assess the inactivation mechanisms of two *L. innocua* strains by the use of both Differential Scanning Calorimetry (DSC) and static microbial inactivation experiments. For that purpose the enthalpy changes obtained from thermograms during dynamic heating are explored *in tandem* with microbial inactivation kinetic calculations.

2. Materials and Methods

Cultures of *L. innocua* strains ATCC 33090 and CCUG 35613 (= ATCC 51742) were stored frozen at -80ºC in cryovials (Microbank, Pro-Lab Diagnostics, Canada). Before each experiment a frozen bead was recovered in Tryptic Soy Broth (Oxoid, Basingstoke, UK) with 0.6% yeast extract (Merck, Darmstadt, Germany) (TSBYE, 10 ml) at 37ºC over night. Inocula were prepared in TSBYE and grown to early stationary phase (growth conditions: 12h/37ºC/150 rpm).

2.1. Inactivation of planktonic cells in capillary glass tubes

For the preparation of capillaries, inoculum was centrifuged (1 mL, 16000 x g, 10 min), supernatant removed and pellet resuspended in TSBYE (1 ml). Static inactivation experiments took place in sterile glass capillary tubes in which a volume of 100 µL of the re-suspended inoculum was inserted using a sterile syringe. Tubes were then sealed by a gas flame and immersed in a water bath (Lauda E300, Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany), at static temperatures from 57ºC to 63ºC. At regular times one capillary was removed from the water bath, placed in an ice-water bath and analysed within approximately 45 minutes. Decimal serial dilutions of the samples were prepared in a TSBYE solution and surface plated on Tryptic Soy Agar (Oxoid, Basingstoke, UK) with 0.6% yeast extract (Merck, Darmstadt, Germany) using a Spiral Plater (Eddy Jet IUL Instruments, Barcelona, Spain). Plates were incubated for 48-96 h at 30ºC and colony forming units were enumerated. Each experiment was performed in duplicate.

2.2. Inactivation of pellet in glass tubes

For the preparation of pellets, cells were harvested by centrifugation of the inoculum (10 mL, 8000 x g, 15 min) and resuspended in an equal volume of a cold buffer (10mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$: 30 mM NH_4Cl). The cells were repelleted and the supernatant was removed. Approximately 70 mg of pellet was transferred to glass tubes (Ø=4 mm) and heated to 62 and 68ºC in a water bath (GR150-S12, Grant Instruments Ltd, Shepreth, UK). Samples were removed at appropriate time intervals, and analysed as described above to obtain the cell concentration. Each experiment was performed in triplicate.

2.3. Differential Scanning Calorimetry

Pellets were prepared as described above. To obtain the thermograms, approximately 70 mg of cell paste (pellet) was transferred into a stainless steel DSC crucible (Medium pressure crucible 120 µl,

Mettler-Toledo GmbH Analytical, Schwerzenbach, Switzerland) which was hermetically sealed. Preheating was carried out in a DSC (DSC 1 system, Mettler-Toledo GmbH Analytical, Switzerland) from 20ºC to end temperature 66-80ºC at a rate of 5ºC/min. After preheating, the samples were immediately cooled to 20ºC (300ºC/min) and scanned during heating from 20 to 120ºC (5ºC/min). The area below the thermogram was integrated between 60-90ºC, using the instrument's software (Star^e Excellence Software, Mettler-Toledo GmbH Analytical, Schwerzenbach, Switzerland).

3. Results & Discussion

3.1. Inactivation of planktonic cells in capillary glass tubes

All data of the inactivation of planktonic cells in capillary glass tubes followed a log-linear decrease. The *D*-value was calculated for each experiment. Log_{10} of *D*-values were plotted versus temperature as shown in Figure 1.

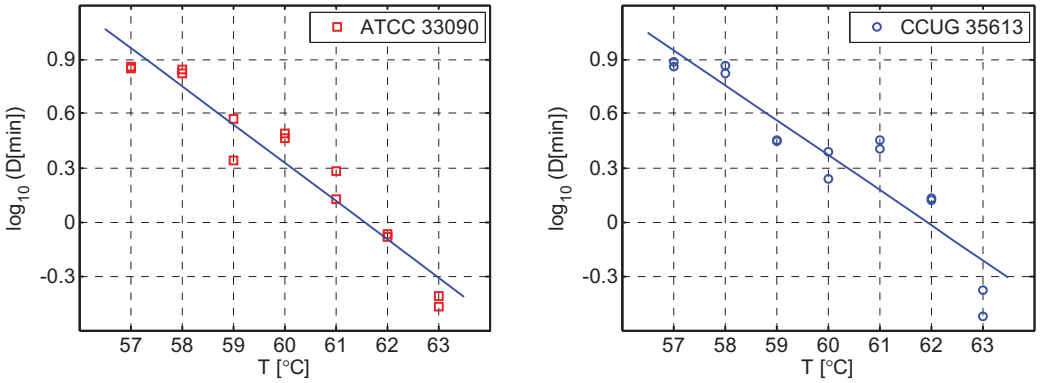

Fig. 1. Log_{10} of *D*-values versus temperature for *Listeria innocua* ATCC 33090 ($R^2 = 0.9306$) and CCUG 35613 ($R^2 = 0.8513$), obtained from isothermal inactivation experiments

Regression analysis (Equation (1) and (2)) was performed (see plots in Figure 1) for estimating the *z*-values of the two strains. The estimated *z*-values were 4.7°C for *L. innocua* ATCC 33090 and 5.2°C for CCUG 35613.

$$Log_{10}D(T) = -0.2121\ T + 13.055 \quad (1)$$

$$Log_{10}D(T) = -0.1927\ T + 11.993 \quad (2)$$

3.2. Inactivation of pellet in glass tubes

Inactivation of the pellet showed very different kinetics compared to inactivation of the planktonic cells (data not shown). From an initial Total Viable Count (TVC) of approximately 1011 CFU in the pellet (approximately 70 mg) the thermal treatments resulted in a log reduction of about 1-2 within the first minute at both temperatures investigated (62 and 68ºC). Subsequent heating, even beyond 10 minutes, did not further reduce the TVC. This phenomenon was not observed in a similar study with pellets of *Esherichia coli* cells in polypropylene tubes [13]. In that study a log-linear inactivation was observed for isothermal heating of pellet which was quite comparable to that of planktonic cells. The

difference could possibly be related to the sample size (15 mg), the species or the water content of the pellet.

3.3. Differential Scanning Calorimetry

The thermograms were obtained during heating from 20° C to 120° C, after dynamic pre-heating to 66, 68, 70, 72, 74, 76, 78 and 80° C at 5C/min. An example of a thermogram, obtained after dynamic preheating to a final temperature of 66° C is shown in Figure 2.

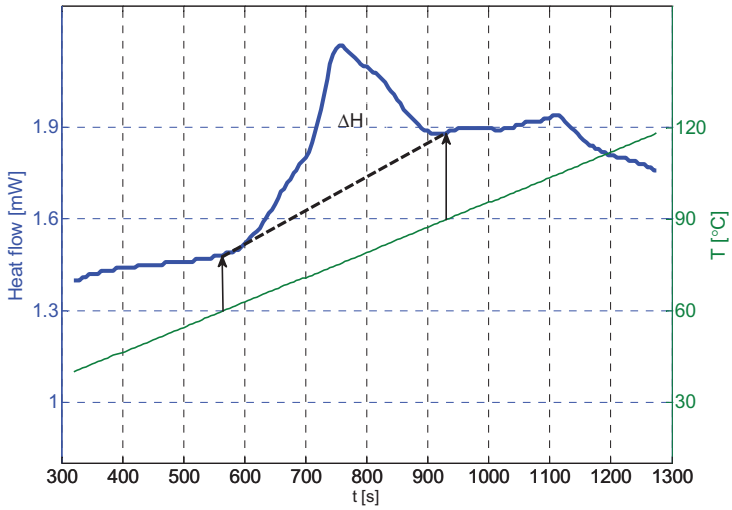

Fig. 2. Plot of typical thermogram (thick line) with preheating to 66ºC and temperature profile (thin line). The area above the thick dashed line and the thermogram is the integrated area in the 60-90ºC region

The most prominent changes in the thermograms due to preheating takes place between 60 and 90ºC, with a peak around 76ºC which can be associated to the denaturation temperature of the 50S subunit and the 70S particle of the ribosome [10]. By integration of the area below the thermogram in this temperature region the remaining enthalpy, ΔH [mJ/mg], can be determined for each dynamic profile. This remaining enthalpy indicates to what degree the sample has absorbed energy, non reversibly, during the pre-heating. In this case it is believed to be a measure of non-reversible denaturation of the ribosome of the Listeria cells [14]. Plotting the remaining enthalpy versus the end-temperature of the dynamic pre-heating profile reveals a linearly decreasing trend with increasing pre-heating temperature (figure 3).

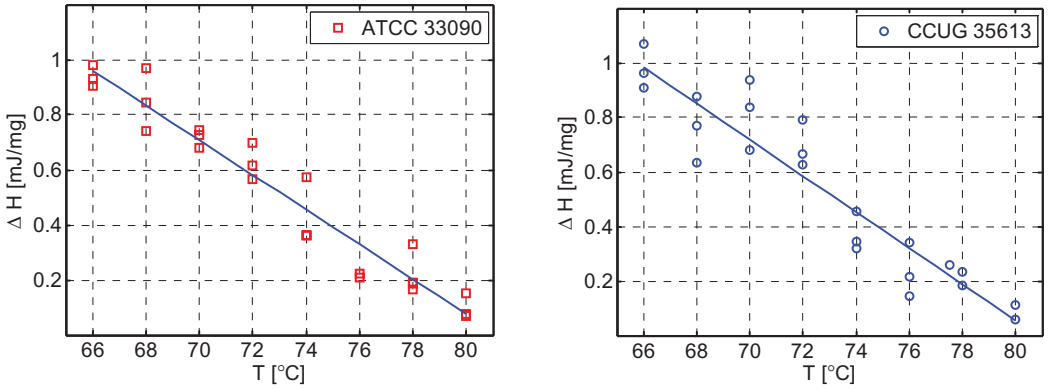

Fig. 3. Enthalpy change in pellets of *Listeria innocua* ATCC 33090 ($R^2 = 0.941$) and CCUG 35613 ($R^2 = 0.894$) during heating from 20-120ºC, as a function of the final temperature of the dynamic pre-treatment profile

This linearly decreasing trend is similar for the two strains investigated. Preheating performed to temperatures below 66ºC does not show any increase in energy uptake during the subsequent scan to 120ºC, as compared to 66ºC. Hence it appears that the first ribosome denaturation takes place during treatment to temperatures higher than 66ºC. It should be noted that what is of main importance for inactivation of microorganisms is not the total denaturation of the ribosome or other active molecules, but the enthalpy difference between the active and inactive states of the system [15].

Based on the inactivation kinetics (D and z values) calculated from the inactivation of planktonic cells of in capillary tubes, the microbial inactivation can be simulated for any temperature profile by using the dynamic form of the linear model (equation (3)). Microbial simulations were performed by describing the temperature evolution during the dynamic pre-treatment of the DSC experiments (equation (4)) and integrating this information into equations (1) or (2) and then equation (3).

$$\frac{d\log_{10} N(t)}{dt} = -\frac{1}{D(T)} \tag{3}$$

$$T = T_0 + r*t \tag{4}$$

where $d\log_{10}N/dt$ is the change in surviving organisms per unit time. $D(T)$ is the value of the decimal reduction at the actual temperature calculated from the equations (1) and (2). T is the actual temperature, T_0 the initial temperature of the pre-treatment (20°C), r the rate of temperature change during pre-treatment, i.e., 5ºC/min, and t the actual time.

Using these equations, the inactivation caused by the dynamic heating profile could be calculated. From this inactivation an isothermal heating temperature giving rise to the same inactivation as the dynamic heating profile within the same time span (t_{final}) was estimated. This is referred to as the equivalent isothermal temperature for the DSC dynamic preheating. The log reduction at these isothermal conditions (ΔN), and the D-value at the equivalent isothermal temperature (D-equivalent) were calculated and are shown in table 1. This table also shows the relation of the equivalent isothermal temperature to the energy uptake as derived from the thermograms at the different pre-treatment temperatures.

Table 1. The dynamic pre-treatment temperatures, the equivalent isothermal temperature and the corresponding log reduction and D-value for Listeria innocua ATCC33090 and CCUG35613

Strain	Dynamic Preheating Final Temperature (°C)	Equivalent Isothermal Temperature (°C)	t_{final} (min)	ΔN ($\log_{10}$ CFU)*	D-equivalent (min)
CCUG 35613	66.0	59.2	9.2	2.77	3.33
	68.0	61.1	9.6	6.75	1.42
	70.0	63.0	10	>10	0.607
ATCC 33090	66.0	59.6	9.2	3.53	2.60
	68.0	61.5	9.6	9.39	1.02
	70.0	63.4	10	>10	0.401

*reduction in CFU in a pellet (approximately 70 mg)

In figure 4 the equivalent isothermal temperature is plotted versus the enthalpy change. With respect to the impact of this treatment (dynamic heating to a final temperature of 66ºC), it can be seen from table 1 and figure 4 that this corresponds to isothermal heating at >59ºC for 9.2 minutes, for both strains. Table 1 shows that this leads to a log 3 reduction (approximately) for both strains.

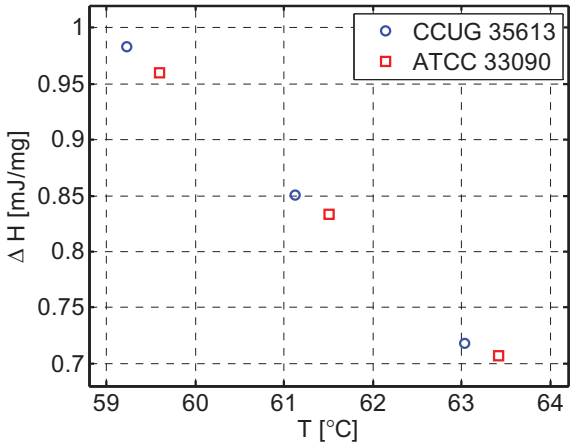

Fig. 4. The equivalent isothermal temperature correlated to the enthalpy change (mJ/mg) in pellets of *Listeria innocua* ATCC 33090 and CCUG 35613 during the DSC-scan to 120ºC

The DSC results indicate no enthalpy change with dynamic preheating to 66ºC. If this phenomenon is independent on the specific form of the cells (pellet, planktonic form) then no denaturation of the ribosome is expected during the studied heating process. This corresponds well to previous findings indicating that mild heat treatment primarily cause inactivation due to cell membrane damage [8].

4. Conclusions

Our results indicate that the denaturation of the ribosomes, as measured by the energy uptake during dynamic heating (5ºC/min) is not the major cause of inactivation for the two strains of *L. innocua* investigated. The relationship between thermal death and the thermographs obtained between 60-90ºC are of interest to further understand the major inactivation mechanism of *Listeria* species. The approach used

in this research enables to link thermodynamic data obtained from dynamic heating profiles with microbial kinetic studies.

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